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Macrolide inactivation gene cluster *mphA-mrx-mphR* adjacent to a class 1 integron in *Aeromonas hydrophila* isolated from a diarrhoeic pig in Oklahoma

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***Objectives*: To characterize a multidrug-resistant *Aeromonas hydrophila* isolate (CVM861) that possesses a high-level macrolide inactivation gene cluster (*mphA-mrx-mphR*), previously only reported in *Escherichia coli*.**

***Methods*: PCR fragment length mapping, gene sequencing and Southern blotting were used to map the *mphA-mrx-mphR* gene cluster and flanking elements in CVM861. Conjugation experiments were done to determine whether the multidrug resistance genetic element was mobile.**

***Results*: The *mphA-mrx-mphR* gene cluster mapped downstream of a class 1 integron and upstream of an *aph(3′)* gene, and was present on a Tn*21*-like element. The gene order determined by sequencing was *intI1-dhfrXII-orfF-aadA2-qacΔE-sul1-orf5Δ178-tnpA-mphR-mrx-mphA*. Horizontal transmission of high-level macrolide resistance from CVM861 to *E. coli* 47011 was inconsistent; however, a composite plasmid possessing the *mphA* gene cluster was transferred at a conjugation frequency of 2.02×10^{-5} per recipient.**

***Conclusions*: An *mphA-mrx-mphR* gene cluster was present downstream of the In2 integron located on a Tn*21*-like transposon in an *A. hydrophila* isolate. Whether this recombination event resulted in the truncation of the *orf5* sequence is unknown. The presence of other resistance genes downstream of the *mphA-mrx-mphR* gene cluster suggests that multiple recombination events have occurred on this genetic element. This is the first known report of the *mphA-mrx-mphR* gene cluster carried by *A. hydrophila* and the first known isolation of this cluster in the United States.**

Keywords: multidrug resistance, Tn*21*, transposons

Introduction

The increased prevalence of multidrug-resistant bacterial pathogens has become a threat to both human and animal health. The clinical failure of antimicrobials in the treatment of infections in food animals results in increased rates of morbidity and mortality; thus affecting both the cost and safety of food. Currently, no single treatment is available that can eliminate the need for antimicrobials in food animal production. Broad spectrum use of antimicrobials has selected for multidrug-resistant bacteria in both human and agricultural venues.[1,2] In many multidrug-resistant bacterial strains, resistance genes have become localized on mobile genetic elements, such as integrons, transposons and broad host range plasmids.[3] Horizontal transfer of mobile genetic elements is considered a significant factor driving the global spread of multidrug-resistant bacteria.[4,5] Furthermore, the linkage of resistance genes allows the persistence of some types of resistance in the absence of selective pressure.[3,6]

Resistance to macrolide antimicrobials, but not lincosamides or streptogramins, can occur as the result of enzymic drug modification.[7] An inducible macrolide phosphotransferase gene cluster that confers high-level resistance to 14-membered ring macrolides has been described in *Escherichia coli*.[8–10] The gene cluster consists of *mphA-mrx-mphR*.[10] MphA is a phosphotransferase that phosphorylates and thus inactivates erythromycin. Mrx is a protein of unknown function, but is required for MphA expression. MphR negatively regulates *mphA* gene expression at the transcriptional level.[11] The presence of the *mphA* gene cluster in *E. coli* has been of little clinical significance, because Enterobacteriaceae are intrinsically resistant to macrolide antimicrobials due to drug efflux transporters.[12]

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Aeromonas hydrophila is a Gram-negative, β-haemolytic fish pathogen and an opportunistic pathogen of humans and warm-blooded animals. *Aeromonas* spp. are most often associated with aquatic habitats[13] and are a primary health concern to the commercial fish farming industry.[14,15] Many are cold tolerant and are considered psychrotrophic food pathogens of fish and other retail meats.[16–18] *A. hydrophila* has become a medically significant pathogen during the past decade and has been considered the source of gastroenteritis in humans in the absence of other pathogens.[19,20] Other clinical illnesses attributed to *Aeromonas* spp. include urinary tract infections, haemolytic uraemic syndrome, wound infections, septicaemia and a variety of respiratory tract infections.[21]

Conjugative transfer of R-plasmids has been described for *Aeromonas* spp. under many experimental conditions,[22–25] including the intestinal tract of swine.[24] The presence of class 1 integrons among aeromonads of aquatic origin has also been described.[22,26] L'Abee-Lund and Sorum[26] noted that class 1 integrons from human pathogens have a greater tendency to contain multiple integron-associated gene cassettes than do those isolated from environmental and fish pathogens.

This study presents a multidrug-resistant *A. hydrophila* isolate (CVM861), isolated from a diarrhoeic pig in Oklahoma, that carries the high-level macrolide resistance gene cluster (*mphR-mrx-mphA*) downstream of a class 1 integron on a Tn*21*-like element. The integron contains trimethoprim/sulfamethoxazole, streptomycin–spectinomycin, quaternary ammonium amine and sulphonamide resistance gene cassettes (*dhfrXII*, *aadA2*, *qacEΔ1* and *sul1*). An aminoglycoside 3′-phosphotransferase [*aph(3′)*] resistance gene is located downstream of the *mphA* gene cluster.

Materials and methods

Bacterial strains

CVM861 was originally isolated from a diarrhoeic pig on a swine farm in Oklahoma by the FDA Center for Veterinary Medicine (CVM), Laurel, MD, USA. The isolate was provided as an unspeciated β-haemolytic isolate from swine and was subsequently identified as *A. hydrophila* in this study. *A. hydrophila* (19570, 23211 and 13444) were obtained from the ATCC (Manassas, VA, USA). Eleven aquatic *A. hydrophila* isolates BSU-B, -C, -E, -G, -I, -J, -K, -L, -M, -N and -O were obtained from the White River (Muncie, IN, USA) in February 2004. *A. hydrophila* KG1 was obtained on site (College Station, TX, USA) from a healthy adult pig in 2002. *A. hydrophila* isolates were grown on blood agar and identified according to EPA Method 1605. The identification as *A. hydrophila* was based on the following criteria: positive oxidase, trehalose and indole fermentation, APE 20E identification (bioMérieux, Hazelwood, MO, USA), and ribotyping.[3] Rifampicin-resistant *E. coli* 47011 was used as a recipient in conjugation studies and was obtained from the ATCC. *E. coli* RP1-EM481 and *E. coli* pTZ3509 were graciously provided by Dr Norihisa Noguchi (Department of Microbiology, Tokyo University of Pharmacy and Life Science, Tokyo, Japan) and have been previously described.[27]

Determination of antimicrobial susceptibility

The antimicrobial MICs were determined by broth microdilution according to methods described by the Clinical Laboratory Standards Institute (CLSI).[28,29] Susceptibility testing was performed with the Sensititre® automated antimicrobial susceptibility system according to the manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH, USA). NARMS panels for Gram-negatives were used in the Sensititre system; the following antimicrobials were assayed: amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftriaxone, cefalotin, erythromycin, gentamicin, kanamycin, rifampicin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline, trimethoprim/sulfamethoxazole and tylosin. The MICs of erythromycin and tylosin were determined manually by broth microdilution in Mueller–Hinton broth. *E. coli* 25922, *E. coli* 35218 and *Pseudomonas aeruginosa* 27853 were used as controls for antimicrobial susceptibility testing and were obtained from the ATCC. Data were interpreted using CLSI breakpoints; when unavailable, breakpoints from the NARMS 2004 Annual Report (NARMS 2004) were used.[30] The MICs for the quality control strains were within the acceptable ranges established by the CLSI.[28,29]

Molecular analysis

Preparation of genomic DNA was done according to manufacturer's instructions using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Plasmid DNA was extracted from tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) overnight cultures using an alkaline lysis method.[31] Standard PCR reactions (50 μL) contained template DNA (100 ng), 25 pmol of each of the selected primers (Table 1), 25 μL of HotStarTaq Mastermix (Qiagen) and water to give the required volume. All reactions were first heated to 95°C for 10 min, followed by 35 cycles of PCR with a denaturation temperature of 94°C (1 min), an annealing temperature of 60°C for primers 20–21 bases in length and 58°C for primers <20 bases in length (Table 1) (1 min) and an extension temperature of 72°C (1 min). For amplicons longer than 1000 bp the extension time was increased to 2 min. Primer combinations used to generate overlapping PCR amplicons for sequencing were as follows: *mphA*-f/*mphA*-r (837 bp); *intI1*-f/3′CS (2460 bp); A1/A3 (1104 bp); A2/A4 (1765 bp); A5/A12 (740 bp); A6/A7 (2078 bp); A10/A3 (1588 bp); A19/A11 (1596 bp); A14/A13 (1069 bp); A15/A16 (835 bp); A6c/A18c (1645 bp); and *mxr*-r/*mphA*-f (1448 bp). Amplicons were purified by the Qiaquick PCR Purification Kit (Qiagen) and submitted for DNA sequencing at the DNA Core Facility in the Department of Veterinary Pathobiology, Texas A&M University, College Station, TX, USA. Sequence comparisons were made using the BLAST program available at the National Center for Biotechnology Information.[32]

Additional primer combinations used to generate PCR amplicons for mapping gene positions were as follows: *intI1*-f/3′CS (2459 bp); 5′CS/3′CS (1912 bp); A2/A12 (2482 bp); *intI1*-f/*intI1*-r (281 bp); 5′CS/A13 (2027 bp); Tn*21*/int-r (1518 bp); *merA*-f/*merA*-r (1232 bp); and A21/A22 (1029 bp).

Bacterial conjugation

Mobility of high-level erythromycin resistance from *A. hydrophila* CVM861 was determined by a solid conjugation method.[33] *E. coli* RP1-EM481 was used as a conjugative positive control donor strain.[34] Rifampicin-resistant *E. coli* 47011 was used as a recipient in all conjugation experiments. CVM861 and *E. coli* RP1-EM481 were grown in TSB containing 256 mg/L of erythromycin overnight at 37°C. *E. coli* 47011 was grown in TSB overnight at 37°C with rifampicin at 32 mg/L. Donor cells (0.05 mL) and recipient cells (0.5 mL) were mixed in 4.5 mL of fresh TSB then collected by filtration on a 13 mm membrane filter. The filter was placed on a tryptic soy agar plate with 5% sheep blood (TSA II 5% SB; Becton Dickinson & Co., Sparks, MD, USA) and incubated at 37°C for 18 h. The cells were collected from the membrane by washing in 1 mL of TSB. The TSB wash from the conjugation experiments were spread-plated on tryptic soy agar (TSA; Difco Laboratories) plates containing rifampicin (32 mg/L) and erythromycin (256 mg/L). Additional conjugation experiments were done substituting streptomycin (32 mg/L) or kanamycin

Table 1. Oligonucleotides used for PCR amplification and DNA sequencing

Primer	Sequence	Orientation and position in accession no. AY522923	Reference
intI1-f	5′CCTCCCGCACGATGATC3′	forward –43/–27 bases[a]	Bass *et al.*[43]
intI1-r	5′TCCACGCATCGTCAGGC3′	reverse 237–221	Bass *et al.*[43]
5′CS	5′GGCATCCAAGCAGCAAGC3′	forward 505–522	Bass *et al.*[43]
3′CS	5′AAGCAGACTTGACCTGAT3′	reverse 2416–2401	Bass *et al.*[43]
mphA-f	5′AACTGTACGCACTTGC3′	reverse 9854–9839	Sutcliffe *et al.*[44]
mphA-r	5′GGTACTCTTCGTTACC3′	forward 9018–9033	Sutcliffe *et al.*[44]
A1	5′CGGCAATGGCGCTGACTACGT3′	forward 3524–3544	this study
A2	5′GATTACGTCTCGCAGGGCGCG3′	forward 4466–4486	this study
A3	5′AACGACGCAGGTAGCAATTGC3′	reverse 4626–4606	this study
A4	5′GCGGGTCGCCGACAGGTAGAA3′	reverse 6230–6210	this study
A5	5′TTCTACCTGTCGCCGACCCGC3′	forward 6210–6230	this study
A6	5′TTACGCATGTGCCTGGAGGAG3′	forward 7161–7191	this study
A7	5′GATGATCGACTGGAGCGAGGC3′	reverse 9238–9218	this study
A10	5′CACCGTGTTTCAATCGACAGC3′	forward 3039–3059	this study
A11	5′GATATCACCCGAGCAGGGGAC3′	reverse 3827–3807	this study
A12	5′GACGATACTCCGCCAATGCGC3′	reverse 6949–6929	this study
A13	5′AATGCGGATGTTGCGATTACT3′	reverse 2531–2511	this study
A14	5′TCGGTCAAGCCGAGCCGCATT3′	forward 1462–1482	this study
A15	5′GGGTTATGAATCTGCATCGCG3′	forward 6772–6792	this study
A16	5′GCGCTTCACCAACCGCGATAC3′	reverse 7606–7588	this study
A19	5′GCCTCACGCGCAGATCACTTG3′	forward 2232–2252	this study
A21	5′TGTAACAGTCACGTCGGTTAT3′	forward 15227–15247	this study
A22	5′GCCCGACATTATCGCGAGC3′	reverse 16255–16238	this study
Tn*21*-f	5′CGGGTTCTGGTCGAAGGTGC3′	forward –1281–1261[a]	Sunde and Sorum[45]
aadA-r	5′TCGGCGCGATTTTGCCGGTTA3′	reverse 2127–2107	Sutcliffe *et al.*[44]
merA-f	5′ACCATCGGCGGCACCTGCGT3′	forward (undetermined)	Bass *et al.*[43]
merA-r	5′ACCATCGTCAGGTAGGGGAACA3′	reverse (undetermined)	Bass *et al.*[43]
A6c	5′CTCCTCCAGGCACATGCGTAA3′	reverse 7191–7161	this study
A18c	5′CCTCACGCCACGAACGCACCG3′	forward 5547–5567	this study
mxr-r	5′TGGAGAGAGTCGCCGTCGTGG3′	forward 8371–8391	this study

[a]Upstream AY522923 sequence origin.

(32 mg/L) for erythromycin. Conjugation frequency was calculated by dividing the number of transconjugants by the initial number of recipients.

DIG-labelled probes

CVM861 genomic DNA was used to generate a Tn*21*-specific 1517 bp amplicon. Purified plasmid DNA from *E. coli* pTZ3509 that contained only the *mphA* and *mrx* genes was used to generate the *mrx-mphA* 1484 bp fragment. These two PCR amplicons were purified and digoxygenin (DIG)-labelled by PCR amplification according to the manufacturer's instructions (Roche Applied Sciences, Indianapolis, IN, USA).

Southern blotting

Southern-blot analysis was performed by the method described by Sambrook *et al.*[31] Genomic DNA from *A. hydrophila* CVM861, *E. coli* JM109 (negative control) and *E. coli* RP1-EM481 (positive control) (10 µg/sample) was digested with the restriction enzymes *Kpn*I and *Sca*I (New England Biolabs, Beverly, MA, USA) and separated on a 0.8% agarose gel. DIG-labelled λ *Hin*dIII DNA (0.01 µg) (Roche Applied Sciences) was used as markers. Following transfer of DNA to the nylon membrane, the membrane was incubated at 49°C for 3 h in EasyHyb solution containing 0.1 mg/L of herring sperm DNA. The membrane was then incubated in hybridization buffer (EasyHyb solution containing the DIG-labelled probe and herring sperm DNA) for an additional 18 h at 49°C. Chromogenic detection of DIG-labelled hybrids was performed using a DIG Labeling and Detection Kit according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN, USA).

EMBL GenBank accession numbers

The GenBank accession number for the *A. hydrophila* CVM861 macrolide phosphotransferase gene cluster and Tn*21* sequence determined for this study was AY522923. Accession numbers used for comparison were as follows: *E. coli* pTZ3509 (AB038042);[27] *E. coli* Tn*21* transposition genes, In2, and mercury resistance operon (AF071413);[40] Tn*21* In2 insertion sequence 1353 (U42226);[35] *Shigella flexneri* Tn*21SF1* (AF188331) (no corresponding publications); *E. coli* Tn*21* dihydrofolate reductase XII (Z21672);[36] and *E. coli mphA* (D16251).[10]

Results

Characterization and localization of the mphA gene cluster on a Tn21-like DNA element

PCR fragment length mapping initially indicated the *mphA* gene cluster was located downstream and in the opposite orientation from a class 1 integron *sul1* gene. A total of 9948 bp

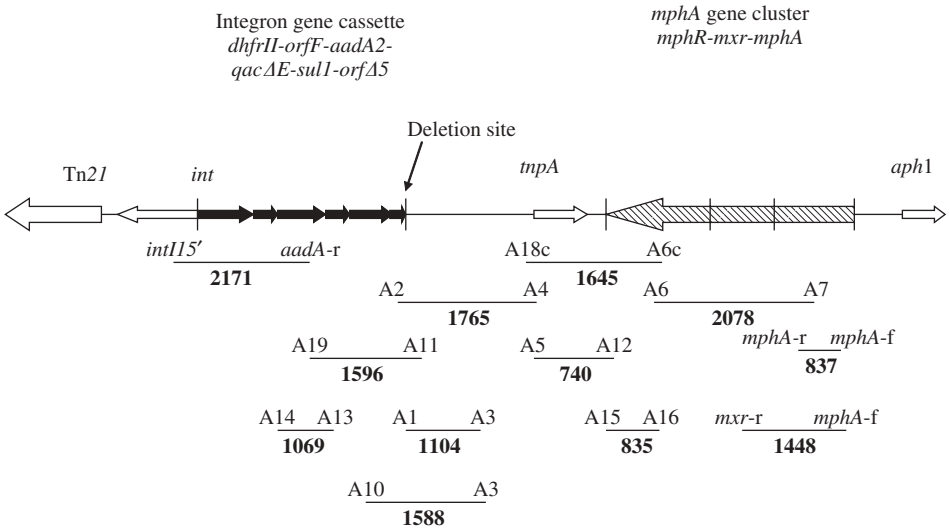

Figure 1. Physical and genetic structure of CVM861 Tn*21*-like transposon (not to scale). The class 1 integron antimicrobial resistance genes are shown as black arrows and the macrolide phosphotransferase genes are shown as a hatched arrow. For simplicity, only PCR amplicons used for sequencing are shown. The numbers in bold represent the sizes of the amplicons in base pairs.

of genomic DNA from CVM861 was sequenced from overlapping PCR amplicons (Figure 1) (EMBL GenBank accession no. AY522923). PCR and sequence analysis of CVM861 genomic DNA revealed the presence of the entire macrolide inactivation gene cluster *mphA-mrx-mphR*. The gene order determined by sequencing 9948 bp was *int-dhfrXII-orfF-aadA2-qacΔE-sul1-orf5Δ178-tnpA-mphR-mrx-mphA*.

The CVM861 *mphR-mrx-mphA* sequence was identical with the pTZ3905 *mphR-mrx-mphA* sequence (GenBank AB038042). Alignment analysis of the CVM861 sequence between the *intI1* gene and the *mphA* gene was very nearly identical with a Tn*21*-like *S. flexerni* transposon, Tn*SF1*, with the exception of a 601 base deletion corresponding to 9324–9924 of the Tn*SF1* sequence. The deletion occurs 978 bases downstream of *orf5Δ178* on the CVM861 sequence. The CVM861 *dhfrXII-orfF* gene sequence was identical with the dihydrofolate reductase *dhfrXII* and *orfF* gene sequence reported on a Tn*21*-like element (GenBank Z21672).

Since the CVM861 sequence was so similar to the Tn*21*-like *S. flexerni* transposon, Tn*SF1*, the presence of Tn*21* on the CVM861 sequence was investigated. A forward Tn*21* primer was used in combination with the integrase reverse primer to determine whether the 5′ portion of Tn*21* was present. The expected 1517 base PCR amplicon was produced. PCR analysis to detect the mercuric reductase gene (*merA*), known to be present on the 3′ end of Tn*21*, was done using *merA* primers and generated the expected 1237 base amplicon. The position of *merA* was not mapped. The presence of *aph(3′)* was determined using primers A21 and A22 that span the region from the 5′ end of *mphA* into the 5′ end of *aph(3′)*. The expected 1028 bp amplicon was produced indicating *aph(3′)* was immediately downstream of the *mphA* gene cluster.

Incidence of mphA and integrase genes among A. hydrophila

Subsequent to the determination that *mphA* was carried by *A. hydrophilia*, additional *A. hydrophilia* isolates were examined for the prevalence of the macrolide phosphotransferase gene. Total genomic DNA from eleven aquatic, one swine and three ATCC *A. hydrophila* isolates was used for PCR screening. Only *A. hydrophilia* CVM861 was positive for the *mphA* gene. To determine whether the *mphA* gene was present in a class 1 integron gene cassette, and to determine the incidence of integrons among the *A. hydrophila* isolates, PCR was done using class 1 integrase gene specific primers; *intI1*-f and *intI1*-r (Table 1). In contrast to the *mphA* gene, the class 1 integrase gene was highly prevalent among the aeromonads tested. Only *A. hydrophila* ATCC 23211 and BSU-B were negative for the class 1 integrase gene in replicate analyses (data not shown).

Antimicrobial susceptibility and mobility resistance determinants

In addition to high-level resistance to erythromycin (≥512 mg/L), CVM861 displayed resistance to amoxicillin/clavulanic acid, ampicillin, gentamicin, kanamycin, spectinomycin, streptomycin, tetracycline, sulfamethoxazole and trimethoprim/sulfamethoxazole (Table 2). CVM861 exhibited resistance to azithromycin at 128 mg/L, but only intrinsic (64 mg/L) resistance to tylosin. Transfer of high-level erythromycin resistance by CVM861 did not occur consistently. No transconjugants were obtained using kanamycin or streptomycin in combination with rifampicin. When transconjugants were obtained they appeared to contain a composite genetic element. The conjugation efficiency of one successful conjugation experiment was 2.02×10^{-5} per recipient to *E. coli* 47011. *E. coli* RP1-EM, the conjugative positive control, transferred high-level erythromycin resistance at a conjugation efficiency of 3.28×10^{-3} per recipient. All ($n = 10$) transconjugants analysed from the successful conjugation described, exhibited a single antimicrobial susceptibility profile that was different from CVM861. This transconjugant phenotype was defined as ARS861-47011. ARS861-47011 lost resistance to trimethoprim/sulfamethoxazole, gentamicin, streptomycin–spectinomycin and sulfamethoxazole. This corresponds to the genes located in the class 1 integron, In2. Additional PCR using the forward Tn*21* primer and the integrase reverse primer confirmed that at least

Table 2. Antimicrobial susceptibility profiles for bacterial isolates used in this study

Isolate	API profile	Antimicrobial susceptibility											
		AMX	AMP	GEN	KAN	RIF	SPT	STR	SXT	TET	ERY	TYL	AZM
E. coli RP1-EM481	1044552	*16*	4	≤1	**>64**	≤0.5	≤8	≤0.5	≤16	**>32**	**≥512**	**128**	**ND**
E. coli pTZ3509	0044552	≤1	≤0.5	≤1	≤8	≤0.5	≤8	≤32	≤16	≤4	**≥512**	**128**	**ND**
E. coli 47011	5044552	8	2	≤1	≤8	**≥512**	≤8	≤32	≤16	≤4	**64**	**64**	2.0
A. hydrophila CVM861	3046134	**>32**	**>32**	**>8**	**>64**	≤1	**>64**	**>64**	**≥512**	**>32**	**≥512**	**64**	**128**
E. coli ARS861-47011	5044552	**>32**	**>32**	≤1	**>64**	**≥512**	≤8	≤0.5	32	**>32**	**≥512**	**128**	**128**
A. hydrophila 19570	7043654	*16*	**>32**	0.25	8	≤0.5	≤8	32 NI	0.25	4	**64**	**64**	**ND**
A. hydrophila 23211	3043655	*16*	**>32**	0.5	8	≤0.5	≤8	32 NI	0.12	4	**64**	**64**	**ND**
A. hydrophila 13444	3042245	8	**>32**	2	8	≤0.5	≤8	32 NI	0.12	4	**64**	**64**	**ND**
A. hydrophila KG1	3046127	**32**	**>32**	0.5	8	≤0.5	≤8	32 NI	0.25	4	**64**	**64**	**ND**
A. hydrophila BSU-B	2043024	*16*	**>32**	1	8	≤0.5	≤8	32 NI	0.12	4	**64**	**64**	**ND**
A. hydrophila BSU-C	2047127	**32**	**>32**	0.5	8	≤0.5	≤8	32 NI	0.25	4	**64**	**64**	**ND**
A. hydrophila BSU-E	3047127	*16*	**>32**	1	8	≤0.5	≤8	32 NI	0.12	4	**64**	**64**	**ND**
A. hydrophila BSU-G	3047524	**32**	**>32**	2	8	≤0.5	≤8	32 NI	2	4	**64**	**64**	**ND**
A. hydrophila BSU-I	3041004	*16*	**>32**	0.5	8	≤0.5	≤8	32 NI	0.5	4	**64**	**64**	**ND**
A. hydrophila BSU-J	6047124	*16*	**>32**	0.5	8	≤0.5	≤8	32 NI	0.12	4	**64**	**64**	**ND**
A. hydrophila BSU-K	1047127	**32**	**>32**	0.25	8	≤0.5	≤8	32 NI	0.12	4	**64**	**64**	**ND**
A. hydrophila BSU-L	1047126	*16*	**>32**	2	8	≤0.5	≤8	32 NI	**>4**	4	**64**	**64**	**ND**
A. hydrophila BSU-M	1045126	*16*	**>32**	0.25	8	≤0.5	≤8	32 NI	0.12	4	**64**	**64**	**ND**
A. hydrophila BSU-N	3047127	*16*	**>32**	2	8	≤0.5	≤8	32 NI	0.25	4	**64**	**64**	**ND**
A. hydrophila BSU-O	1047134	*16*	**>32**	0.5	8	≤0.5	≤8	32 NI	0.12	4	**64**	**64**	**ND**

AMX, amoxicillin/clavulanic acid; AMP, ampicillin; GEN, gentamicin; KAN, kanamycin; RIF, rifampicin; SPT, spectinomycin; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; ERY, erythromycin; TYL, tylosin; AZM, azithromycin; ND, not determined; NI, not interpretable.
Intermediate susceptibility is indicated by italics; resistance is indicated by values in boldface. MICs are in mg/L.

part of In2 had been deleted. The presence of *mphA* and *aph(3′)* in ARS861-47011 was confirmed by PCR.

Southern-blot analysis

Overlapping PCR amplicons suggested the *mphA* gene cluster was on the Tn*21*-like element. However, to confirm this hypothesis Southern blots were done using CVM861 genomic DNA. *Kpn*I cuts the Tn*21* upstream of the Tn*21* primer site within the *tnpA* gene at positions 1533–1529 of the EMBL GenBank accession no. AF071413 sequence. *Sca*I cuts the *Shigella* Tn*21* downstream of the *mphA* gene within the β-lactamase (bla_{TEM-1}) gene of the EMBL GenBank accession no. AF188331 sequence at position 21781. The predicted restriction fragment length of the *Kpn*A and *Sca*I digest with CVM861 DNA was 19 745 bp. Because an exact sequence for the *E. coli* RP1-EM481 Tn*21* derivative transposon was not known, the exact size of the *Kpn*A and *Sca*I digested fragment could not be predicted. However, both the Tn*21* and *mphA* probes detected a fragment ~23 kb in length on the RP1-EM481 digested DNA. A single band of ~20 kb was detected from CVM861 digested genomic DNA using both the Tn*21* and *mphA* probes (data not shown); this indicated the *mphA* gene cluster resides on a Tn*21*-like element as predicted.

Discussion

The *mphA-mrx-mphR* gene cluster localized on a Tn*21*-like element from a human clinical *E. coli* isolate has been described and characterized by Noguchi *et al*.[10,11,27,37] The Tn*21*-like element described by Noguchi *et al*.[37] was located on a mobilizable plasmid. A similar Tn*21*-like transposon sequence Tn*SF1*, carried by *S. flexneri*, has been entered into GenBank with no corresponding publication. Genotypic characterization of *A. hydrophila* CVM861 revealed the presence of an identical *mphA-mrx-mphR* gene cluster, also on a Tn*21*-like transposon. The gene order determined by sequencing was *intI1-dhfrXII-orfF-aadA2-qacΔE-sul1-orf5Δ178-tnpA-mphR-mrx-mphA*. The *mphA-mrx-mphR* gene cluster mapped downstream of a class 1 integron (In2) and upstream of an *aph(3′)* gene. Integron-associated gene cassettes can integrate at secondary insertion sites downstream of the 3′ conserved region of integrons. However, the 59 bp region located on the 3′ end of gene cassettes was not present on the *mphA*, *mrx* and *mphR* gene sequences analysed in this study. Additionally, most gene cassettes contain a single gene with the corresponding 59 bp region.

The *dhfrXII*, *orfF* and *aadA2* on CVM861 Tn*21* were present as gene cassettes in the variable region of the CVM861 integron. The *qacΔE*, *sul1* and *orf5Δ178* genes were part of the 3′ conserved region of the integron. Several antimicrobial resistance gene families have been reported within the confines of class 1 integrons; these include genes that encode resistance to sulphonamides (*sul*), trimethoprim/sulphonamides (*dhfr*), aminoglycoside adenyltransferases (*aadA*), chloramphenicol (*cat*), β-lactamases (*oxa*), erythromycin (*ere*) and quaternary ammonium amines (*qac*).[26,36,38,39] Aminoglycoside adenyltransferases and dihydrofolate reductases are the most prevalent integron-associated gene cassettes in the variable region of integrons, and they are often adjacent to each other as was observed for CVM861.[39]

Repeated transposition events can lead to deletions of necessary transposition genes; ultimately, resulting in immobility of the integron or transposon.[5,40] In2, the integron localized on Tn*21*, is transposition-defective due to the insertion sequence (IS*1353*) in transposition genes.[35] IS*1353* is downstream of the normal *orf5*. An apparent transposition event resulting in the integration of the *mphA* gene cluster may have caused the partial deletion of *orf5*. The CVM861 sequence diverges from the In2 sequence at the *orf5*Δ*178* terminus. Therefore, the IS*1353* element may have been deleted or may reside downstream of the 3′ region sequenced in this study. Further evaluation would be necessary to determine whether IS*1353* is present further downstream on the CVM861 transposon. A 601 bp deletion that distinguishes the CVM861 sequence from the *S. flexneri* Tn*SF1* sequence occurs 978 bp downstream of the *orf5*Δ*178* terminus. This may suggest the 601 bp deletion occurred after the integration of the DNA containing the *mphA* gene cluster. The structural similarity of the *S. flexneri* Tn*SF1* to the CVM861 Tn*21*-like transposon suggests a common origin, probably originating in Asia.

The antimicrobial resistance genes identified on the CVM861 Tn*21*-like transposon correspond to the phenotypic resistance profile exhibited by the isolate except for tetracycline. Resistance to tetracycline suggests the presence of tetracycline resistance genes possibly on a Tn*10*-like element; however, this was not investigated during this study. Tn*21* was originally identified on NR1 a 94.5 kb broad host range plasmid isolated from *S. flexneri* in Japan.[40] The original NR1 structure contained two transposons, Tn*21* and Tn*10*. The prototypic Tn*10* carries tetracycline resistance genes.[40]

Although aeromonads are intrinsically resistant to β-lactam antibiotics, the $bla_{\text{TEM-1}}$ gene may be present downstream of *aph(3′)* on the CVM861 Tn*21*-like transposon. The *Sca*I restriction endonuclease site used for Southern-blot analysis was positioned downstream of the $bla_{\text{TEM-1}}$ gene on the *S. flexneri* Tn*21*-like element. The presence of the *Sca*I site on the CVM861 Tn*21*-like element was hypothetical. Digestion with *Kpn*I alone produced a smear on the Southern blot, whereas, co-digestion with *Sca*I and *Kpn*I produced the predicted 19 745 bp fragment, suggesting the presence of the $bla_{\text{TEM-1}}$ gene on the CVM861 Tn*21*-like element. Both the Tn*21* probe and the *mphA-mrx* probes detected the same 19 745 bp fragment, thus confirming linkage of the *mphA* gene cluster to the Tn*21*-like element.

Horizontal transmission of high-level macrolide resistance occurred randomly from CVM861 to *E. coli* 47011. One successful conjugation experiment produced *E. coli* transconjugants at a conjugation frequency of 2.02×10^{-5} per recipient. The bacterial colonies produced were *E. coli* and not nalidixic acid-resistant *A. hydrophilia*, suggesting the CVM861 Tn*21*-like element was on a mobilizable plasmid and that a recombination event was necessary for horizontal transfer of high-level macrolide resistance. This observation is consistent with that described by Noguchi *et al.*[37] The *E. coli* transconjugant produced (ARS861-47011), exhibited a different antimicrobial resistance phenotype from CVM861. ARS861-47011 lost resistance to trimethoprim/sulfamethoxazole, gentamicin, streptomycin–spectinomycin and sulfamethoxazole. This corresponds to the genes located in the class 1 integron, In2. Additional PCR confirmed that at least part of In2 had been deleted. The susceptibility of ARS861-47011 and PCR analysis suggests that *aph(3′)*, $bla_{\text{TEM-1}}$ and the *mphA* gene cluster remained intact.

The presence of the *mphA-mrx-mphR* gene cluster on the CVM861 Tn*21*-like element could have easily gone undetected had it not been specifically investigated. Gram-negative isolates are not normally tested for macrolide susceptibility due to intrinsic resistance. Genotypic characterization of 15 other *A. hydrophila* isolates suggested the gene cluster was not common to aeromonads. However, class 1 integrons were prevalent among the aeromonads examined in this study. The integron-associated gene cassettes of the other aeromonads were not sequenced; however, susceptibility to most of the antimicrobials tested may suggest the presence of single or empty gene cassettes. Schmidt *et al.*[15] reported a 45% incidence of class 1 integrons among aeromonads at Danish trout farms. Of the isolates containing class 1 integrons 16% did not contain integron-associated gene cassettes. Rosser and Young[39] reported a 3.6% incidence of class 1 integrons among 3000 Gram-negative isolates from an estuarine environment. Roughly half of the integrons characterized did not contain integron-associated gene cassettes; and some of the integrons lacked *sul1*, *qacE*Δ*1* or both in the 3′ conserved region. Variation from the prototypic integron structure may reflect the lack of selection pressure in an estuarine environment.[39] The aquatic aeromonads used in this study originated from the White River in Muncie, IN, USA, where medically important antibiotic selection pressure was unlikely. However, additional genotypic characterization would be necessary to determine whether the prototypic integron structure is intact among the White River aeromonads.

Treatment of enteric pathogens in swine commonly includes the use of broad-spectrum antimicrobials.[41,42] Tylosin is a 16-membered ring macrolide antimicrobial used in food animal production. However, MphA does not effectively use tylosin as a substrate, and did not confer high-level resistance to tylosin in this study. The lack of direct selective pressure to maintain the *mphA* gene cluster in food production applications would eventually lead to point mutations and deletions in the sequence. Other selection pressures, including mercury resistance, could lead to the persistence of Tn*21* but not necessarily individual genes. The *mphA* gene cluster that integrated onto Tn*21* appears to have remained functional during interspecies transfer among *S. flexneri, E. coli* and *A. hydrophila*; as well as during global dissemination between Asia and the United States. It would be interesting to examine isolates carrying the *dhfrXII* gene to determine whether the *mphA* gene cluster is commonly found in association with *dhfrXII*, especially since the origin of the isolates from Houston, Texas and Okalahoma are relatively close geographically.

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Transparency declarations

None to declare.

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